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13. ABSTRACT *(Maximum 200 Words)*

Genes and signaling pathways implicated in EMT and the invasiveness of breast cancers include FGF, Notch and T-box and Ets family transcription factors. Studies were performed relating to each of these pathways and factors. One aim goal was to examine the relationship between Notch signaling and the Brachyury T-box transcription factor. Through cloning and characterization of this gene it was shown that Brachyury is not a target of Notch signaling and does not function in mesoderm formation, and therefore EMT. A second T-box family member implicated in breast cancer progression, Tbx2, was cloned and characterization initiated. A polyclonal antibody was generated and antisense oligonucleotides synthesized to assay Tbx2 function during EMT. Additionally, three other T-box family members were cloned; homologues of Tbx4/5, Tbx6 and T-brain/Eomes.

To look at direct effectors of EMT, a series of experiments looking at members of the Rho GTPase family were initiated. Homologues of Rho, Rac and cdc42 were cloned and dominant negative/constitutively active forms of these proteins generated. The goal of this research is to provide an *in vivo* insight into the mechanism whereby adhesions are assembled and disassembled during EMT and metastasis and thereby identify potential targets for therapeutics aimed at preventing the spread of breast cancers.

14. SUBJECT TERMS
epithelial-mesenchymal transition, metastasis, adhesion, Notch, FGF, T-box Transcription factors, Brachyury, Ets, Rho GTPases

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Table of Contents

Introduction:

Epithelial-mesenchymal transitions (EMTs) are indicative of cancer metastasis and correlate with poor patient survival. These transitions, however, are a normal part of animal development. The EMT that occurs during mesenchyme formation in the sea urchin is highly representative of the transition that occurs during cancer metastasis in that identical signaling pathways and effector molecules are involved. This transition has been well characterized in the sea urchin and thus provides an *in vivo* model system to further elucidate the changes that cells undergo during EMTs. This study sought to identify signaling pathways and molecular correlates to EMT. In particular studies include; examination of Notch and FGF signaling pathways, members of the T-box and Ets transcription factor families and proteins directly involved in the transition such as the Rac, Rho and cdc42 small G-proteins. The goal of these studies is two-fold; first to enhance the knowledge on endogenous EMTs in order to have a basis of comparison for those occurring during metastasis and second, to identify new genes involved in the process and therefore provide potential targets for new therapies aimed at blocking cancer progression.

Body:

During this annual period, significant progress was made on the research proposed. This progress is summarized below.

Most time during this period was spent addressing Specific Aim 2, 'To characterize the role of FGF and Notch signaling during epithelial-mesenchymal transitions'. Months 1-18 in the 'Statement of Work' were allocated to study Notch signaling, T-box genes, and the relation between the two. Several significant observations were made. MMTV induced tumorigenesis previously identified the INT3 gene as a locus for viral insertion (Gallahan and Callahan 1987, 1997). The INT3 gene incodes a mammalian Notch homologue, Notch4. Viral insertion generates a truncated protein consisting of only the intracellular portion of the receptor, and thus, a constitutively active signal. In the sea urchin, a similar form of the Notch protein (LvN^{act}) when expressed results in an increase in the number of migratory mesenchyme cells (Sherwood and McClay 1999).

The first aspect of this specific aim was to characterize Brachyury in the sea urchin as a possible target of Notch signaling as implicated by previous studies (Corbo et al. 1998). A sea urchin homologue of Brachyury was cloned (Fig 1) and its expression characterized by Northern blot (Fig 2) and by generation of polyclonal antisera against recombinant protein (Fig 3). Brachyury was found to not localize to mesoderm tissue as was suggested by *in situ* observations by another laboratory (Harada et al. 1995). Further, a dominant interfering construct was generated by fusing the DNA binding domain of Brachyury to the repression domain of the *Drosophila* engrailed gene (Fig 4A). This construct when expressed in sea urchin eggs had no effect on mesenchyme formation, rather, it blocked the movements of the endoderm during gastrulation (Fig 4B). The endoderm tissue was unable to invaginate into the blastocoel and form the embryonic gut. Thus, Brachyury was determined to not be a target of Notch signaling, nor to be involved in mesoderm formation and therefore not a useful target for therapeutics aimed at blocking EMT. The work, however, is significant in that it shows Brachyury functions in gastrulation movements of the endoderm. Significant adhesive and cytoskeletal changes are necessary for such movements to occur and therefore Brachyury may be important in coordinating these changes. This work has been submitted to *Development* and is under review.

The next aspect of this specific aim was to identify and characterize other T-box genes in the sea urchin. In particular, the function of Tbx2 was of interest to this study. Recently, Tbx2 has been implicated in breast cancer progression. In breast cancers, amplification of the chromosomal region 17q22-q24 is often observed. Four genes in this region are overexpressed in breast cancer cell lines, one of which is Tbx2 (Barlund et al. 2000). Additionally, Tbx2 was identified in a screen of genes possessing the ability to confer immortality to fibroblast cells and found to be overexpressed in a subset of primary human breast cancers (Jacobs et al. 2000). During this annual period, a sea urchin homologue of Tbx2 was cloned and its expression characterized by Northern blot (Fig 5). Antiserum has been generated against recombinant protein and current studies are underway to localize the protein during development. Additionally, an antisense

morpholino oligonucleotide has been generated in order to perturb Tbx2 function and assay its role in development. The hope is that an effect on EMT or adhesion will be observed. The identification of a T-box transcription factor overexpressed in breast cancers may be significant in that this family of genes could participate in the progression of the disease. To that end, during this annual period we have also cloned homologues of several other T-box genes in the sea urchin. PCR clones of Tbx4/5, T-brain/Eomesodermin and Tbx6 have been identified and currently cDNA libraries are being screened to isolate full length clones of these genes. Future work will characterize the spatial and temporal expression of these genes and determine their function during normal development and EMT.

Work on FGF signaling during this period also progressed. It was decided that injection of a dominant negative FGF1 receptor mRNA from *Xenopus* would not be the clearest means to assay FGF signaling in the sea urchin and its role in EMT as a *Xenopus* construct might not recapitulate endogenous FGF signaling in the urchin. Therefore, this experiment was eliminated from Aim 2 and instead efforts were focused on isolation of endogenous sea urchin FGF receptors for characterization and functional assay. To that end, potential FGF receptors were and are currently being sought through a combination of degenerate PCR and cDNA library screening with othologous genes from other organisms.

It is the goal of this study to isolate one or more sea urchin FGF receptors and characterize them during the current annual period and thereby complete the original goals of Specific Aim 2. In addition to the FGF studies, continued work on Specific Aim 2 during the current annual period will involve the characterization of other T-box genes in the sea urchin that might play a role in EMT and therefore breast cancer metastasis and progression.

EMT has been extensively studied in *in vitro* models where epithelia in culture can be induced to undergo EMT after treatment with various cytokines (Aaronson 1991). Cells necessarily must abrogate these cell-cell interactions in order to gain the ability to migrate from the plane of an epithelium. *In vitro* systems are informative but not truly indicative of what is occurring *in vivo* during either normal development or in a pathological context. The goal of the studies described in this proposal was to characterize an *in vivo* model of EMT in order to extend studies, by comparison, to metastasis and therefore to cancer spread. To that end we have also initiated a series of experiments aimed at looking at potential effectors of EMT rather than solely inductive signals. During this annual period, sea urchin homologues of the Rho GTPases Rho, Rac and cdc42 were cloned. These proteins are intimately associated with the remodeling of adhesive junctions and the actin cytoskeleton (Braga 2000, Schmitz et al. 2000). Rho GTPases are necessary for the formation and disassembly of cell-cell contacts *in vitro* (reviewed in Schmitz et al. 2000). Experiments have been designed to ascertain the functions of these GTPases during *in vivo* EMT in the sea urchin embryo. These studies will hopefully corroborate the *in vitro* observations and perhaps provide a model system in which potential therapies derived to prevent EMT can be tested. Dominant negative and constitutively active forms of each of the sea urchin homologues have been

constructed and are currently being expressed in the embryo to assay their function during EMT. These studies will be continued during the current annual funding period in hopes of further understanding their effects on EMT.

Additionally experiments in the current annual period will continue studies on Ets factors, a family of winged-helix transcription factors thought to play a role in the progression and invasiveness of breast cancers. It is our goal during this annual period to perturb Ets expression in the sea urchin and characterize the effect on EMT. Additionally, a subtractive screen for Ets downstream genes will be performed.

Key Research Accomplishments:

- Cloning of sea urchin Brachyury homologue
- Northern characterization of Brachyury expression
- Anti-Brachyury polyclonal antibody generated
- Brachyury expression pattern characterized
- Brachyury function characterized through expression of a dominant interfering construct
- Determination that Brachyury is not a target of Notch signaling and is not involved in mesoderm formation and therefore not an aspect of EMT
- Cloning of sea urchin Tbx2 homologue
- Northern characterization of Tbx2 expression
- Anti-Tbx2 polyclonal antibody generated
- Cloning of sea urchin Tbx4/5 homologue
- Cloning of sea urchin T-brain/Eomesodermin homologue
- Cloning of sea urchin Tbx6 homologue
- Cloning of sea urchin Rho homologue
- Cloning of sea urchin Rac homologue
- Cloning of sea urchin cdc42 homolohue
- Generation of dominant negative and constitutively active Rho, Rac and cdc42 constructs to characterize function

Reportable Outcomes:

- Manuscript submitted to *Development* and under review, 'The Role of Brachyury 'T' During the Gastrulation Movements of the Sea Urchin, *Lytechinus variegatus*'
- Invited Seminar – October 2000, 'Developmental Biology of the Sea Urchin' Woods Hole, MA
- Poster Presentation – December 2000, 'American Society For Cell Biology Annual Meeting' San Fransisco, CA
- Antibodies generated: Anti-Brachyury polyclonal serum, Anti-Tbx2 polyclonal serum

Conclusions:

Genes and signaling pathways implicated in EMT and the invasiveness of breast cancers include FGF, Notch and T-box and Ets family transcription factors. During the past funding period, studies were performed relating to each of these pathways and factors. A goal of the study was to examine the relationship between Notch signaling and the Brachyury T-box transcription factor. Through cloning and characterization of this gene it was shown that Brachyury is not a target of Notch signaling and does not function in mesoderm formation, and therefore EMT, rather functioning in the gastrulation movements of the endoderm. A second T-box family member implicated in breast cancer progression, Tbx2, was cloned from the sea urchin and characterization initiated. A polyclonal antibody was generated and antisense oligonucleotides synthesized to assay Tbx2 function during EMT. Additionally, three other t-box family members were cloned in this period – homologues of Tbx4/5, Tbx6 and T-brain/Eomes.

To look at direct effectors of EMT, a novel series of experiments were initiated looking at members of the Rho GTPase family. Sea urchin homologues of Rho, Rac and cdc42 were cloned and dominant negative/constitutively active forms of these proteins generated. Studies expressing these altered constructs were begun to assay their function in adhesion and EMT. The overall goal of this research is to provide an *in vivo* insight into the mechanism whereby adhesions are assembled and disassembled during EMT and metastasis and thereby identify potential targets for therapeutics aimed at preventing the spread of breast cancers.

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Figure 1

A

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ATG CCT GCA ATG AGC GCC GAC GCT CTC CGC GCA CCG ACC TAC AAC GTT TCG CAT CTA CTC 60
 M   P   A   M   S   A   D   A   L   R   A   P   T   Y   N   V   S   H   L   L
ACC GCC GTG CAA TCA GAA ATG AAC CGA GGC AGT GAG AAG GGA GAC CCT AGC GAG AAG GGG 120
 T   A   V   Q   S   E   M   N   R   G   S   E   K   G   D   P   S   E   K   G
CTC AAA GTC AGG CTA GAG GAC GTG GAA CTA TGG AAG AAA TTT CAC AAA TTG ACC AAC GAG 180
 L   K   V   R   L   E   D   V   E   L   W   K   K   F   H   K   L   T   N   E
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 M   I   V   T   K   S   G   R   R   M   F   P   V   L   S   A   S   I   A   G
CTC GAC CCG AAC TCC ATG TAC TCA ATA CTG CTC GAC TTT TCC GCT GCT GAC GAC CAC CGC 300
 L   D   P   N   S   M   Y   S   I   L   L   D   F   S   A   A   D   D   H   R
TGG AAG TAC GTC AAT GGG GAG TGG GTA CCC GGC GGG AAG CCC GAC GGC TCG CCT CCG ACC 360
 W   K   Y   V   N   G   E   W   V   P   G   G   K   P   D   G   S   P   P   T
ACC GCT TAC ATC CAC CCC GAC TCG CCA AAC TTT GGC GCT CAC TGG ATG AAA CAA GCC GTC 420
 T   A   Y   I   H   P   D   S   P   N   F   G   A   H   W   M   K   Q   A   V
AAC TTC AGC AAA GTC AAG CTC TCC AAC AAG CTC AAC GGA AGC GGA CAG GTA ATG TTG AAT 480
 N   F   S   K   V   K   L   S   N   K   L   N   G   S   G   Q   V   M   L   N
TCC CTT CAC AAG TAC GAA CCA CGA ATC CAC ATC GTA CGG GTA GGA GGC AGG GAG AAA CAG 540
 S   L   H   K   Y   E   P   R   I   H   I   V   R   V   G   G   R   E   K   Q
AGA TTG GTC GGA AGT TAT TCG TTC GCA GAA ACT CGC TTT ATT GCC GTT ACA GCA TAC CAA 600
 R   L   V   G   S   Y   S   F   A   E   T   R   F   I   A   V   T   A   Y   Q
AAT GAA GAC ATA ACA CAA CTC AAA ATT AAA TAC AAC CCA TTC GCC AAG GCT TTT CTC GAC 660
 N   E   D   I   T   Q   L   K   I   K   Y   N   P   F   A   K   A   F   L   D
ATA AAA GAC AAA AAT GAA GGG CAT GAT CTC TTT GAT GAT GTA CAC GAT TCT CAG GGT TCG 720
 I   K   D   K   N   E   G   H   D   L   F   D   D   V   H   D   S   Q   G   S
AAA TAT CCA CAA TTC GGT GGC TGG TTT CTG CCG GGC TCG GGA GCG TTC GGC CCC TCA CCG 780
 K   Y   P   Q   F   G   G   W   F   L   P   G   S   G   A   F   G   P   S   P
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 H   Q   F   G   P   S   L   G   L   P   S   H   A   G   C   E   R   Y   G   G
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 L   R   G   H   R   T   S   P   Y   P   P   P   P   Y   H   Q   K   Y   G   A
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 H   Q   P   N   L   G   S   T   A   H   G   M   G   P   G   G   S   V   H   P
TCC ACC GCC GCG GTC ACG ACC GCA AAC TCG ACC GAG ACG CAT GCG CAG AGT CAG TCC GTC 1260
 S   T   A   A   V   T   T   A   N   S   T   E   T   H   A   Q   S   Q   S   V
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 M   A   P   I   S   V   P   I   S   S   A   A   G   H   D   M   S   G   S   P
GCC GCG GTG CAT TGC CAT CAA ACG GGC TAC CAT GAC TCG TAC CAT GGG GAC ATC ACC GGA 1380
 A   A   V   H   C   H   Q   T   G   Y   H   D   S   Y   H   G   D   I   T   G
AGC GCT GTT GGT CTG GAT ATC AGC GGC TAT GGA GCC TGC CAT ACA TCA AAC CAT CAT CAT 1440
 S   A   V   G   L   D   I   S   G   Y   G   A   C   H   T   S   N   H   H   H
AAT TCT CCG CAT CAA CAG TTG GTC GGG AAA AGT GTT TCA TCT TGG AAC CCC TTA ACA CCG 1500
 N   S   P   H   Q   Q   L   V   G   K   S   V   S   S   W   N   P   L   T   P
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 P   S   V   .
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B

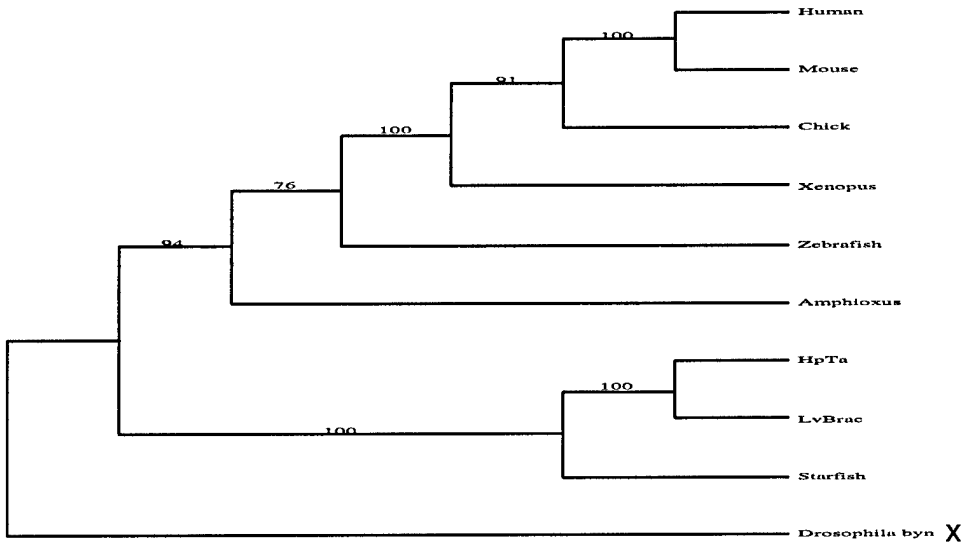

Figure 2

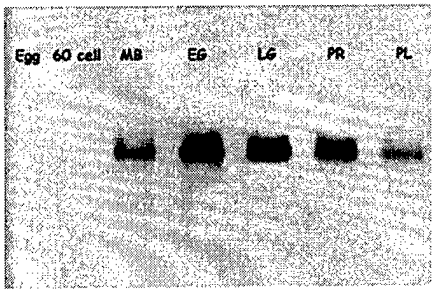

Figure 3

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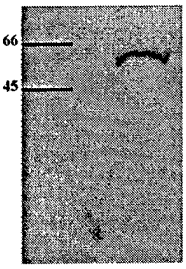

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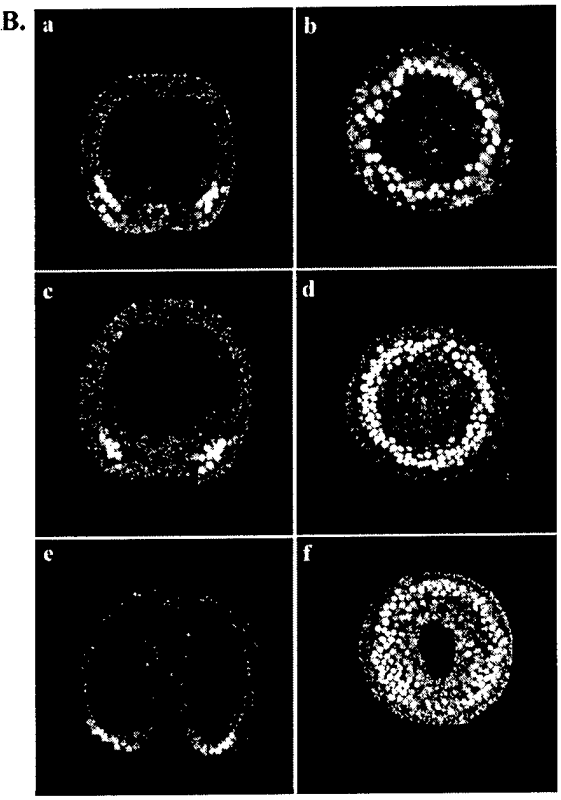

Figure 4

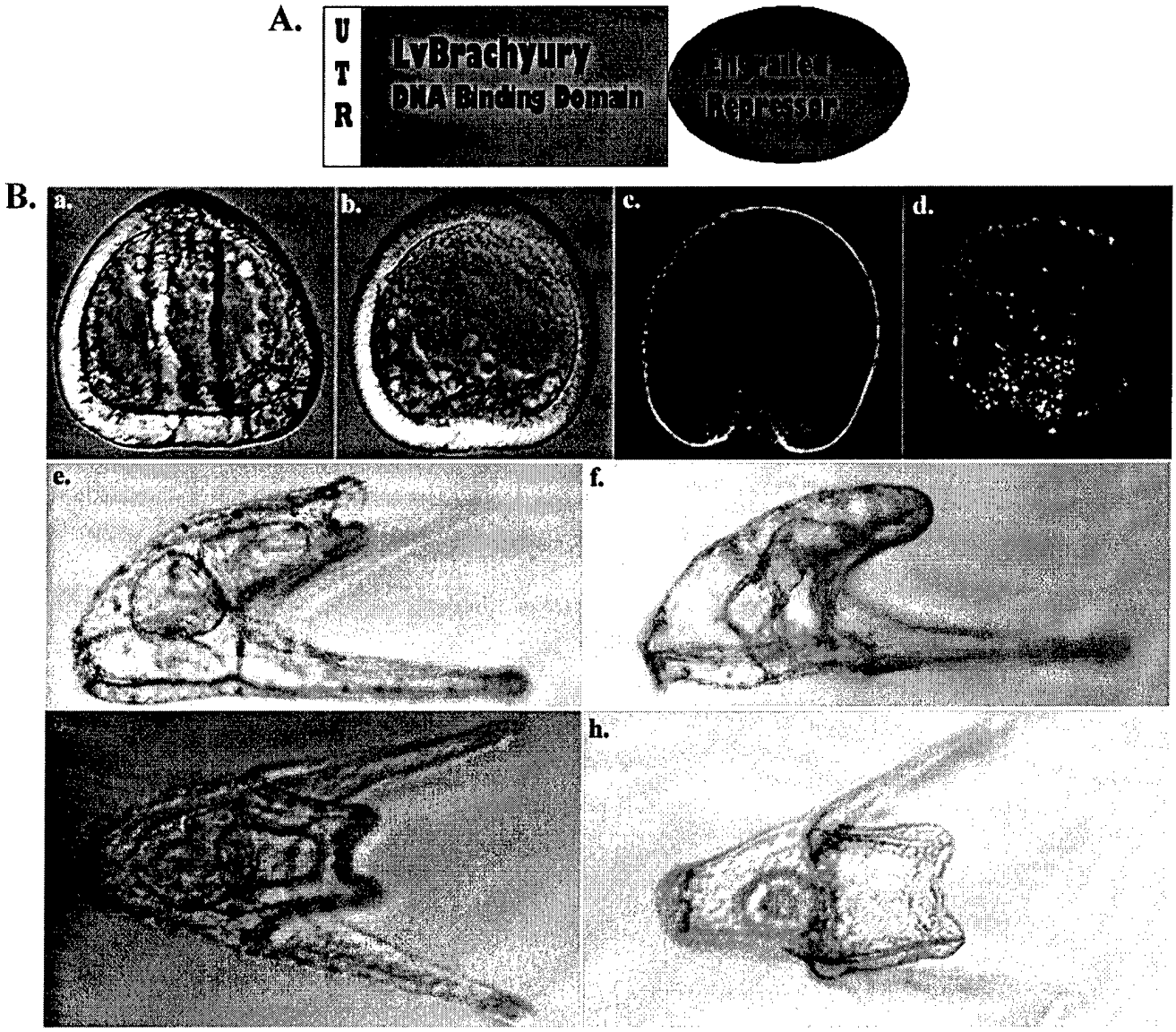

Figure 5

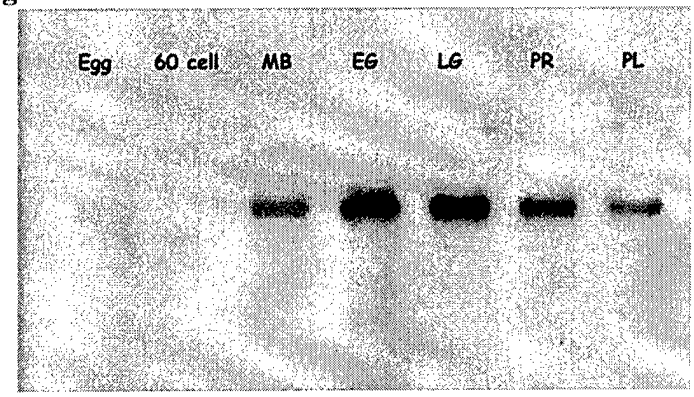

FIGURE LEGENDS:

Figure 1 – (A) Nucleotide and predicted amino acid sequences of LvBrac. Sites used for degenerate PCR primers are underlined. (B) Phylogenetic tree of LvBrac and *Brachyury* orthologues from other animals. Bootstrap values indicated on nodes.

Figure 2 – Developmental northern blot of LvBrac expression. 3ug/lane of poly(A)$^+$ RNA was loaded as calculated by OD_{260} and loading verified by probing the blot with a *Lytechinus pictus* ubiquitin fragment (data not shown). Egg; 60 cell; MB, mesenchyme blastula; EG, early gastrula; LG, late gastrula; PR, prism; PL, pluteus larva.

Figure 3 – (Panel A) Western analysis of mid-gastrula protein extracts (1000 embryos) using affinity purified polyclonal LvBrac serum. A single immunoreactive band of approximately 56 kDa appeared, in agreement with the predicted open reading frame size of LvBrac. (Panel B) LvBrac displays a dynamic expression pattern in the vegetal hemisphere during sea urchin gastrulation. (a,,c,e) Cross sections and (b,d,f) vegetal views. (a,b) LvBrac first appears at mesenchyme blastula stage in a circumferential region of nuclei within the vegetal plate, (a) Cross section of an embryo where the lateral extent of the expression domain can be viewed. (b) Vegetal view where the full diameter of the expression is shown. (c,d) Slightly older early gastrula embryos where the archenteron is beginning primary invagination. (e,f) Late gastrula embryos where the archenteron has nearly reached the top of the blastocoel and therefore completed gastrulating. In (a-f), the area of expressing cells appears to remain constant as tissue has passed through the blastopore and invaginated into the blastocoel.

Figure 4 – Perturbation to LvBrac function via microinjection of mRNA encoding a LvBrac/Drosophila-Engrailed protein. (A) Schematic representation of the LvBrac-EN mRNA construct. (B) Nomarski images and endomesodermal marker staining of control and LvBrac-EN expressing embryos. (a) Appearance of control embryos injected with glycerol (22 hpf). (b) Phenotype of LvBrac-EN injected embryo (22hpf). Note the presence of migrating PMCs (arrowhead) and the smaller gut invagination relative to the control embryo depicted in (a). (c,d) Apical LvNotch expression (c) and SMC-1 expression (oblique cross section, d) in LvBrac-EN injected embryo indicating that endoderm and secondary mesoderm have been specified properly. (e-h) Nomarski images of 48 hpf embryos. (e) Control glycerol injected embryo with normal tripartite gut. (f) High level LvBrac-EN mRNA injected embryo, note the smaller tripartite gut compared to that in (e). (g) Stomodaeum formation in control glycerol injected embryo. (h) High level LvBrac-EN injected embryo lacking a stomodaeum.

Figure 5 – Developmental northern blot of LvTbx2 expression. 3ug/lane of poly(A)$^+$ RNA was loaded as calculated by OD_{260} and loading verified by probing the blot with a *Lytechinus pictus* ubiquitin fragment (data not shown). Egg; 60 cell; MB, mesenchyme blastula; EG, early gastrula; LG, late gastrula; PR, prism; PL, pluteus larva.